Anaerobic Phenotype Microarray Method for Knockout Mutant Comparison

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VIMMS Virtual Institute for Microbial Stress and Survival — Berkeley Lab — Miami University — University of Missouri — Diversa — Oak Ridge National Laboratory, Managed by UT Battelle for the Department of Energy — University of Oklahoma — Berkeley — http://vimss.lbl.gov/

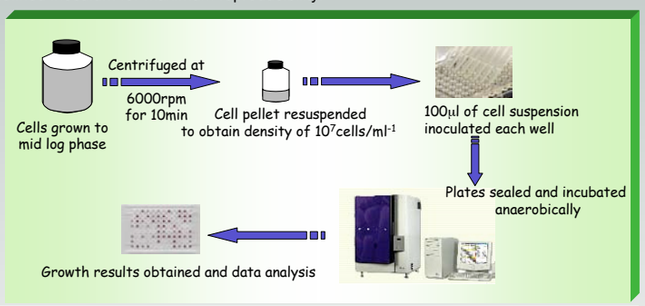
Abstract

Phenotype Microarray (PM) has been developed for the high throughput and rapid assessment of phenotypic responses of microbes to approximately 2,000 metabolites and chemicals under aerobic conditions. Previously in our lab, a method was developed for PM under anaerobic conditions. In the present work we describe a method of inoculum standardization of anaerobes to ensure repeatability of results between replicate runs. Our tests were conducted with the sulfate reducing bacterium *Desulfovibrio vulgaris* strain *Hildenborough* in a defined lactate sulfate medium. For optimization of results, several factors were tested that included growth phase of inoculum having the greatest capability for growth after inoculation, optimal centrifugation times at 6000 g for highest retention of cell pellet, optimal inoculum concentration of resuspended cells as determined by AODC which was compared to OD at 600nm and %T. Our results show that standardization was achieved as demonstrated by repeatability of growth data between biological replicates of *D. vulgaris* in the PM. The application of the anaerobic PM was tested in 2 different studies with a wild type DvH and a single crossover sensor histidine kinase mutant strain of *D. vulgaris* with a potentially interesting phenotype under salt stress. The differential expression patterns of wt *D. vulgaris* and the mutant strain of *D. vulgaris* were compared.Osmotic sensitivity to NaCl and KCl was increased in the mutant strain with inhibition of growth above 3% as compared to 6% and 5% with the wt. No protection of the mutant was conferred by the addition of osmoprotectant. In another test, the mutant strain was used for the novel application of PM technology to investigate phenotypic expression of an organism under stressed conditions. In this study, anaerobic PM of the mutant strain under osmotic stress was generated with 250mM NaCl vs 250mM KCl and compared with the expression pattern of the organism under non stressed conditions. The mutant strain amended with 250mM KCl had greater resistance to osmotic stress up to 10% NaCl and greater resistance to200mM sodium benzoate and 100mM sodium nitrite.

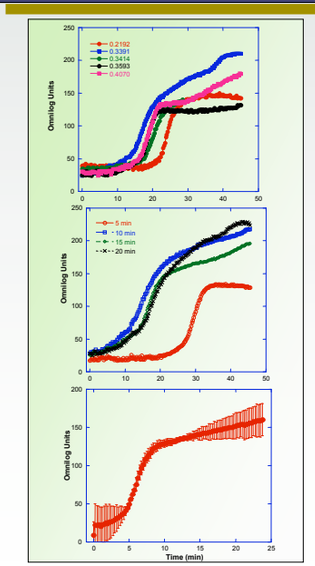
Phenotype Microarray (PM)

The Omnilog system generates a high-throughput and rapid phenotype microarray (PM) of a bacterium of interest. It is possible to investigate phenotypic expression on a wide variety of substrates. Approximately 2,000 assays are run simultaneously to include catabolic and biosynthetic metabolites, ions for osmotic effects, pH, toxic metals and a variety of inhibitory and stimulatory chemicals.

Our group has adapted the system for anoxic incubation of SRBs -specifically *Desulfovibrio* and *Desulfomicrobium* species. Inoculum standardization has been developed to ensure defined inoculum for maximum reproducibility.



Inoculum Standardization



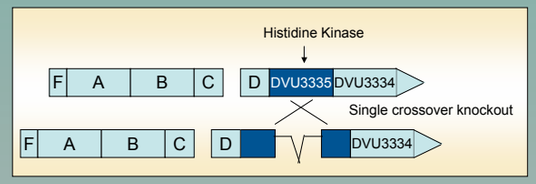
Optimal growth of a 100ul volume of SRB in defined lactate-sulfate medium containing iron is 10^8 cells/ml correlating to mid log phase growth of the SRB with a 10% inoculum. Stationary phase and early log phase cells do not generate high enough final yield to be detected by CCD camera of the Omnilog system

Cells are centrifuged to remove excess medium prior to resuspension in appropriate PM medium. Optimal centrifugation time and speed were established to generate a bacterial cell pellet that could be easily resuspended and homogenized and not result in cell death

Standardized inoculum successfully yields consistent growth patterns of strain DvH in the PM plates with multiple biological replicates.

Plot represents average of 5 biological replicates with std deviation. Of each biological replicate n=96

Mutant Generation (DvAM88) and Expected Phenotype



The Kdp two-component system in bacteria consists of an inducible high affinity transporter of K^+ ions encoded by genes in the *kdpABC* operon where *kdp* is induced by low concentrations of K^+ and repressed by high concentrations of K^+

Single cross over method was used to knock out DVU3335 the histidine kinase present upstream of the response regulator of the *kdp* operon.

The resulting knockout DvAM88 is expected to have a deactivated *kdp* system.

PM Characterization of DvAM 88

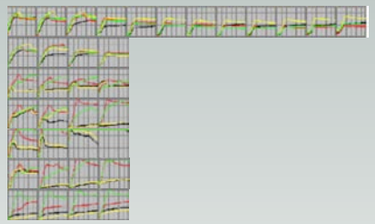
PM overview of growth characteristics of DvAM88 show differences compared to wt

Pretreatment of DvAM88 with salts produced differences between mutant vs wt

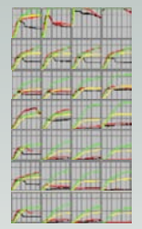
No Stress:
black /yellow =wt
red/green=DvAm88

Salt Stress:
black=wt 250mM NaCl
red = wt 250mM KCl
yellow=DvAM88 250mM NaCl
green=DvAM88 250mM KCl

- NaCl 5-10%
- KCl 3-6%
- $MnCl_2$
- K_2CrO_4
- $NiCl_2$
- Trifluoroperazine
- CCCP

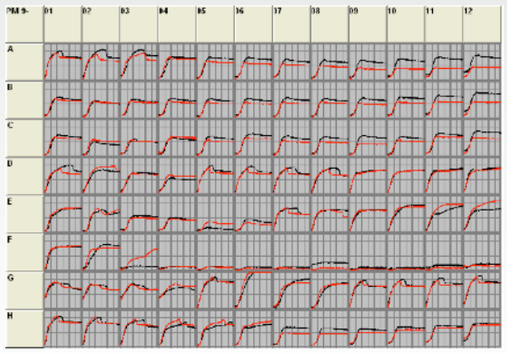


- $NiCl_2$
- Moxalactam
- Tylosin
- K_2CrO_4
- EDTA
- Sodium azide
- Guanidine HCl

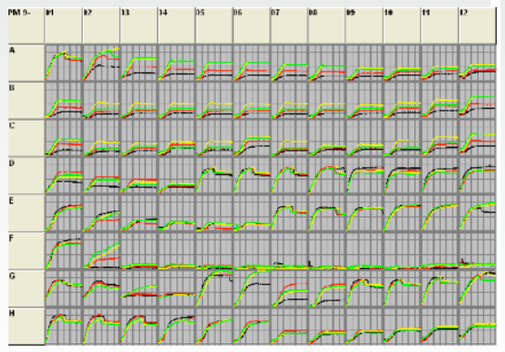


Osmotic and ionic strength expression patterns were compared for DvAM88 and wt utilizing the array of substrates provided on PM Plate #9. This plate was used to test the expression patterns generated from no pretreatment vs salt pretreatment

As expected, phenotypic expression of DvAM88 closely matches wt response. Pretreatment of DvAM88 with salts produced differences between mutant vs wt. These differences have been further explored for confirmation.

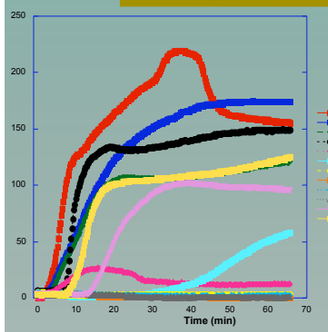


No Stress:
black =wildtype
red=DvAM88

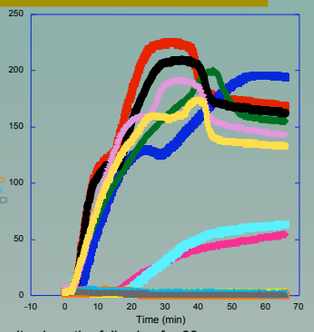


Salt Stress:
black=wt 250mM NaCl
red = DvAM88 250mM NaCl
yellow=wt 250mM KCl
green=DvAM88 250mM KCl

MT Salt Stress verification assays



Results show the following for wt:
50% growth with 250mM NaCl
25% growth with 500mM NaCl
0% growth with 750mM salt

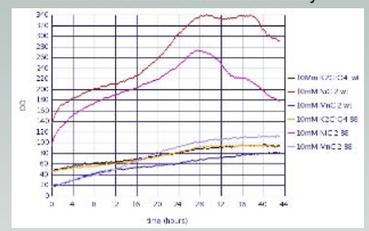


Results show the following for 88:
~100% growth with 250mM KCl and NaCl
25% growth with 500mM KCl and NaCl
0% growth with 750mM salt

DvAM88 appeared to have lower sensitivity to salt especially in the case of K+ ion

Wt shows a lag when exposed to tolerable levels of salt before growth commences but DvAM88 is able to recover faster. Addition of GB cannot rescue wt with 750mM salt but can rescue DvAM88

Metals Utilization Verification Assay



Differences were noted in the expression patterns of wt and DvAM88 on several metals substrates under non stressed and salt stressed conditions.
Non stressed verification assays were performed to test the PM substrates as prepared in our laboratory with replicates in a 100ul 96 well format These verification assays do not support the differences detected in growth by the PM method

Biological Conclusions

- A mutation in the histidine kinase of the kdp system is expected to effect DvH under hypo-ionic conditions- specifically low K+ - however, an interesting aspect of DvHAM88 phenotype was discovered to hyper-ionic stress when DvAM88 was pre-treated with KCl
- Based on these results we conclude that DvAM88 has an inactivated K+ channel allowing it to be more resiliant to K+

PM Conclusions

- The PM array serves the requirement of overviewing the growth phenotype of a strain under predicted and unpredicted conditions
- The PM was successfully adapted for the anaerobic SRB DvH and successfully used for the comparative assessment of the phenotypic expression of wt vs mutant
- We introduce the novel application of the PM for screen of phenotypic expression of a strain under a combination of stressed vs non-stressed conditions

Acknowledgement

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